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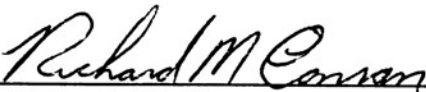
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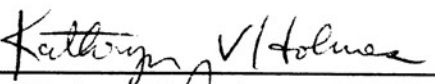
Title of Thesis: **"The Effects of Different Fixatives and Fixation Time on the Extraction and PCR Amplification of RNA from Paraffin Embedded Tissue"**

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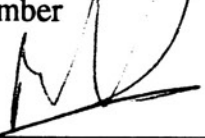
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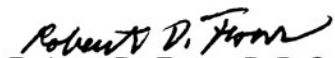


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ABSTRACT

Title of thesis: The Effects of Different Fixatives and Fixation Time on the Extraction and PCR Amplification of RNA from Paraffin Embedded Tissue

LCDR Robert D. Foss, D.D.S., Master of Science, 1993

Thesis directed by: LTC Richard M. Conran, M.D., Ph.D., Assistant Professor, Department of Pathology.

A number of reports have indicated the theoretical possibility of performing PCR on RNA recovered from paraffin embedded tissue (PET). This would have broad ranging implications for research on archival human tissues from a variety of diseases. Although, it is established that RNA in PET has undergone significant degradation, the specific contributions of different fixatives and fixation times to this degradation are not known. A variety of mouse tissues were fixed immediately after sacrifice for either 2, 8 or 24 hours in either formalin, Omnifix II, or Carnoy's fixative. A fourth set of tissues was fixed in the same fixatives for 2 hours after a 24 hour postmortem interval. Spleen, muscle, liver and brain were processed and embedded in paraffin. RNA was extracted from deparaffinized cubes of tissue using a modified proprietary technique. Samples were quantitated and evaluated for purity spectrophotometrically. RNA was reverse transcribed using a random hexamer primed reaction. PCR amplification for cDNAs of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT) was then performed. The PCR products were Southern blotted and probed with specific radiolabelled oligonucleotides. The extraction technique developed provided consistent yields of RNA. GAPDH amplification indicated the presence of DNA contamination in the RNA preparations. Contamination was eliminated

by brief DNase I treatment, but only very limited evidence of intact GAPDH mRNA sequences was found. Amplifiable HPRT mRNA sequences were detected in 9/16 samples fixed in Omnifix II, 7/16 samples fixed in Carnoy's fixative and 3/16 formalin fixed samples. These samples were both DNase treated and untreated. Because of primer selection to preclude amplification of genomic HPRT, DNase treatment did not alter the results. Thus, HPRT represents the control system of choice for the evaluation of RNA in PET. Immediate fixation for 8 hours yielded the most positive samples. Forty percent of the amplifications for HPRT were positive, 19% for formalin fixation and 50% of samples fixed in Omnifix II or Carnoy's fixative. Better preservation of RNA using non-formalin based fixation was observed. The techniques described provide a rapid, uniform and reproducible method of obtaining RNA from PET for molecular analysis.

THE EFFECTS OF DIFFERENT FIXATIVES AND FIXATION TIME
ON THE EXTRACTION AND PCR AMPLIFICATION OF
RNA FROM PARAFFIN EMBEDDED TISSUE

By

Robert D. Foss, D.D.S.

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TABLE OF CONTENTS

	Page
COPYRIGHT STATEMENT	ii
ABSTRACT	iii
ACKNOWLEDGMENTS	vi
LIST OF TABLES.....	viii
LIST OF ILLUSTRATIONS	ix
INTRODUCTION	1
MATERIAL AND METHODS.....	11
RESULTS	25
DISCUSSION.....	42
CONCLUSION	48
BIBLIOGRAPHY	49

LIST OF TABLES

	Page
Table 1. PCR amplification of RNA extracted from PET	6
Table 2. Oligonucleotides used in this investigation	9
Table 3. Abbreviations and designations used.....	12
Table 4. PCR cycling parameters used for GAPDH and HPRT.....	21
Table 5. RNA yields from PET	28
Table 6. A_{260}/A_{280} ratios of RNA extracted from PET	30

LIST OF ILLUSTRATIONS

	Page
Figure 1	14
Figure 2	26
Figure 3	31
Figure 4	34
Figure 5	36
Figure 6	39
Figure 7	40

INTRODUCTION

The ability to predictably recover sufficient undegraded RNA from paraffin embedded tissue (PET) to act as a template for RNA-polymerase chain reaction (RNA-PCR) amplification has broad implications in molecular pathobiology. RNA amplification, through cDNA intermediaries, is of particular interest because of the relationship between mRNA levels and gene expression. Potential applications of such technology include the study of oncogene expression, RNA viruses and characterization of inflammatory or neoplastic lesions by cytokine profiling (Samoszuk et al., 1992). Although amplification of DNA recovered from PET is currently performed on essentially a routine basis, RNA amplification has not yet achieved a comparable status (Wright and Manos, 1990). Recently, however, a number of studies have indicated the potential for the PCR amplification of RNA obtained from archival material (Jackson et al., 1989; Jackson et al., 1990; von Weizsäcker et al., 1991; Ben-Ezra et al., 1991; Stanta and Schneider, 1991; Finke et al., 1993). If this technique proves practical, it has research implications that cannot be overestimated (Greer et al., 1991). This is because of the potential for retrospective analysis of the tremendous volume of pathologic material that exists archivally stored in repositories.

PCR is an extremely powerful technique that has revolutionized the discipline of molecular biology. PCR permits the amplification of specifically targeted sequences of DNA. Once amplified, this DNA can be sequenced, Southern blotted, cloned or otherwise manipulated. Briefly, complimentary sense and antisense oligonucleotide primers flanking the DNA sequence of interest are added to a mixture of template DNA. Also contained in this mix are the appropriate salts, buffers, deoxyribonucleotide triphosphates and a heat stable

DNA polymerase. This reaction mixture is then cycled through three temperature phases; denaturation, annealing and extension. First, the high temperature denaturation phase melts the double stranded DNA, then, upon cooling, the primers will anneal to their complimentary sequences on the separated strands. Lastly, the polymerase extends the primers downstream. The distance separating the ends of the two primers is the length of the amplified product. As this thermal cycling is repeated in the presence of excess primer, the number of copies of the targeted sequence increases geometrically. It is estimated that amplifications in the range of 10^7 - 10^{11} fold can be achieved (Templeton, 1992). The size of the sequence being amplified varies, but products in the 100 to 3000 base pair (bp) range are typical. PCR can also be performed on RNA if the reaction is preceded by a reverse transcriptase (RT) step to produce a DNA strand complementary to the RNA. This step may or may not be directly coupled to the PCR. Using PCR, extremely rare sequences can be detected in a heterogeneous mix of nucleic acids. PCR is ideally suited for the study of infrequently occurring nucleic acid sequences such as mRNAs because of its specificity, sensitivity, simplicity and cost effectiveness.

PCR amplification of nucleic acids recovered from PET was initially demonstrated with DNA (Impraim et al., 1987). This technique is regularly used in a wide variety of research and clinical applications ranging from the identification of oncogene amplification (Frye et al., 1989) to virus typing (Resnick et al., 1990). It is well established that histologic processing of tissue, fixation in particular, does have degradative effects on DNA. These effects are primarily manifested in terms of the length of extracted DNA fragments (Goelz et al., 1985). This may impact on the suitability of a particular tissue specimen for PCR analysis, since as the mean fragment length of the DNA in a sample decreases, so do the chances of a successful amplification (Impraim et al.,

1987). These effects have been evaluated both qualitatively and quantitatively. Greer and colleagues (1991) analyzed the effects of varying fixatives and fixation times on the suitability of PET extracted DNA for PCR. In their study 10% neutral buffered formalin and acetone provided the best results, allowing consistent amplification of DNA sequences up to 1327 bp in length after as long as 24 hours of fixation. Progressively less satisfactory results were obtained with fixatives of increased acidity or containing mercuric chloride such as Carnoy's, Zenker's and Bouin's. Fixatives such as alcoholic formalin, alcoholic formalin-acetic acid, paraformaldehyde, and Clarke's fixative gave intermediate results. Low pH fixation alone did not necessarily correlate with a poor result (Greer et al., 1991). Mean fragment length of DNA recovered from PET varied from 0.1 kbp for Bouin's fixative to 2-5 kbp for acetone and formalin. Similar, but not identical results were obtained by Jackson et al., (1990). This group was able to amplify a 142 bp fragment of DNA from PET. Once again, results were dependent on time and the nature of the fixatives. PCR was successful with tissue fixed in formalin, Carnoy's and Bouin's fixatives, but not in paraformaldehyde or mercuric chloride based fixative. However, in contrast to the study by Greer et al., (1991), Jackson's group, (1990) observed that Carnoy's reagent provided better DNA preservation and recovery than formalin, both in DNA size and amount. Formalin fixation was observed to cause a 40 fold reduction in the amount of recoverable DNA compared to fresh tissue. Interestingly, retention of unfixed tissue for up to seven days at room temperature prior to fixation had a negligible effect on DNA yield in this study. In the aforementioned studies, optimal isolation of DNA was dependent upon digestion of the tissue samples with proteinase K. Additional DNA purification did not significantly affect the outcome of amplification reactions (Greer et al., 1991). Ben-Ezra et al., (1991) similarly compared fixatives and fixation times

with respect to the subsequent extraction of DNA suitable for use as a PCR substrate. Their results are in conflict with the two prior studies cited. This group observed the best results, in terms of an amplifiable 250 bp fragment, with ethanol and Omnifix (a commercially available, ethanol based fixative) fixation. Formalin and acidic or mercuric chloride-based fixatives were noticeably less successful at DNA preservation. The poor results obtained with formalin, with only 3/20 amplifications successful, run counter to other published observations (Greer et al., 1991; Jackson et al., 1990). This may be due to the fact that Ben-Ezra et al., (1991) obtained substrate material for amplification by simple boiling of deparaffinized tissue sections, omitting a proteinase K digestion used by the other authors. This may fail to isolate DNA sequestered by crosslinked proteins. It has also been suggested that proteinase K digestion eliminates PCR inhibitors that may be present (An and Fleming, 1991). According to these results, inhibitors are not factors with alcohol based fixation.

RNA species are highly labile due to the presence of ubiquitous endogenous and exogenous ribonucleases and are generally thought to require fresh or frozen tissues for meaningful analytic studies (Naber et al., 1992), but recent investigations that have paralleled those performed on DNA indicate that RNA recovered from PET can indeed be used as a PCR substrate. Jackson and coworkers, (1989) were the first group to report successful amplification of RNA from PET. A 194 bp and a 245 bp segment of the measles genome from tissue blocks over 12 years old were amplified. Subsequently, several groups have been able to amplify a number of RNA species ranging in length from 75 bp to 230 bp following extraction from PET. In the investigations by Jackson et al., (1989) and von Weizsäcker et al., (1991), RNA recovery was based on a proteinase K digestion of deparaffinized tissue sections followed by phenol/chloroform extraction and alcohol precipitation. This procedure was

modified by Stanta and Schneider, (1991) to include guanidinium thiocyanate in the digestion step. An even more complex procedure combining these steps has been reported by Finke et al., (1993). Examination of illustrated Southern blot or ethidium stained gel results from these studies appears to confirm amplification of the desired cDNA, since the primers used were designed to differentiate the amplification of genomic DNA from cDNAs. The targeted sequences and results of these studies are summarized in Table 1.

DNA contamination appears to present a problem in PET derived RNA, regardless of the extraction technique used. DNA contamination was present in RNA obtained either by digestion followed by chloroform/phenol extraction (von Weizsäcker et al., 1991) or by simple boiling (Ben-Ezra et al., 1991). The illustrations from the study by Ben-Ezra and colleagues, (1991) indicate significant contamination of RNA by genomic DNA. An additional study by Rupp and Locker, (1988) estimated the level of DNA contamination at 5% of total nucleic acids in RNA extracted from PET with proteinase K digestion followed by lithium chloride precipitation.

The effects of fixation on the physical state of RNA are similar to those observed with DNA. Although Ben-Ezra and coworkers, (1991) unexpectedly reported better PCR results with RNA than with DNA, especially in Omnifix-fixed tissues, they may actually have been amplifying genomic DNA pseudogenes (Finke et al., 1993). Northern blot analysis of purified extracted RNA indicates preservation of fragments, on average, in the 200 bp range following formalin or paraformaldehyde fixation. Paraformaldehyde provided slightly better preservation (Stanta and Schneider, 1991). This range of preservation is consistent with the size of amplified target sequences in other studies. A newly described acetone based fixation/embedding technique, the AMeX method, is purported to provide excellent preservation of DNA (Sato et al., 1991). However,

<u>STUDY</u>	<u>TARGET RNA</u>	<u>PRODUCT SIZE (bp)</u>	<u>FIXATION</u>	<u>POSITIVE RESULTS</u>
Jackson et al., 1989	Measles Genome	194/245	Not Stated	4/4
von Weisäcker et al., 1991	Albumin mRNA	230	Formalin	10/10
Ben-Ezra et al., 1991	β -actin mRNA (pseudogenes?- see text)	154	Formalin	14/20
			Omnifix	17/20
			Ethanol	19/20
			Zenker's	6/20
			Bouin's	6/20
			B-5	3/20
Stanta and Schneider, 1991	Retinoblastoma gene mRNA	75	Formalin	35/38
Finke et al., 1993	Porphobilinogen deaminase mRNA	127	Formalin	5/7
	Adolase A mRNA	181		5/7

Table 1. PCR amplification of RNA extracted from PET. Summary of published studies.

RNA extracted from AMeX fixed tissue shows only marginal improvement in integrity over RNA obtained from formalin fixed tissue, when Northern blot results from the work of Rupp and Locker, (1988) are compared with those of Sato et al., (1991). In both of these studies RNA was deemed acceptable for slot- or dot-blot analysis, but PCR was not attempted.

On the basis of the current literature, it may be concluded that amplification of RNA recovered from fixed and processed tissue specimens represents a potential practical application of PCR technology. However, in order for this technique to advance to the status of a clinical application, further study and refinement are necessary. Confirmatory studies characterizing the optimal fixation parameters for the preservation and subsequent extraction of RNA are one element. Earlier studies indicate, without consensus, that of the routinely available histologic fixatives, either 10% neutral buffered formalin (Greer et al., 1991), or Omnifix/ethanol (Greer et al., 1991; Ben-Ezra et al., 1991), or Carnoy's fixative (Jackson et al., 1990) are most likely to provide the best results. Comparison of these three is a reasonable course of action in evaluating the effects of fixation on RNA integrity.

The ability to amplify RNA sequences from PET suggests the need for a reproducible internal control standard that can be extracted and amplified in parallel with the RNA of interest. A control RNA serves multiple purposes. Used appropriately, it should indicate that both the cDNA reaction and the PCR have been accomplished without error and that the target tissue contains amplifiable RNA. The latter aspect can only be addressed using an internal control. In certain cases, for example, if the sequence of interest is derived from a cDNA and the intron-exon structure of the gene is not known or if the existence of intronless pseudogenes cannot be excluded, it is valuable to have a means of detecting contaminating genomic DNA in the prepared sample (Stanta and

Schneider, 1991; Dveksler et al., 1992). Under ideal circumstances such controls have served as standards for relative quantitation of gene expression (Dveksler et al., 1992).

For RNA-PCR, controls are typically based on housekeeping gene mRNAs (Perfetti et al., 1991). Housekeeping genes are constitutively expressed in all tissues. They tend to serve basic metabolic functions in the cell and are highly conserved throughout evolution. Therefore, they often share significant sequence homology and organization from species to species (Kim et al., 1986). The ideal control message is one whose expression in a particular tissue is constant across a wide range of physiologic conditions, but not necessarily constant from one tissue type to another. This is especially important if the aim is to devise a system in which relative levels of gene expression can be compared. Of the earlier studies cited, only β -actin has an established precedent for use as a control. However, β -actin is not without drawbacks as a control since it is subject to transcriptional regulation under a number of physiologic conditions resulting in varying expression levels.

Two systems meeting these requirements, which include sense and antisense oligonucleotide primers and oligonucleotide probes, have been employed successfully as controls in a variety of gene expression experiments (Murray et al., 1990; Svetic et al., 1991; Dveksler et al., 1992). These oligonucleotides are shown in Table 2. The first of these primer/probe sets amplifies and detects the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. This glycolytic enzyme has been found to be constitutively expressed by all tissues thus far examined. Because of the evolutionarily conserved nature of GAPDH, this oligomer set performs equally well in several different species, including mice and humans (Dveksler et al., 1992). The amplified product obtained by the PCR reaction is 195 bp, which falls in the range reported in the

GAPDH:

SENSE PRIMER	5'-CCATGGAGAAGGCTGGGG	18mer
ANTISENSE PRIMER	5'-CAAAGTTGTCATGGATGACC	20mer
PROBE	5'-CTAAGCATGTGGTGGTGCA	19mer

PCR PRODUCT: 195 BASE PAIRS

REFERENCE: Dveksler et al., 1992

HPRT:

SENSE PRIMER	5'-GTTGGATACAGGCCAGACTTTGTTG	25mer
ANTISENSE PRIMER	5'-GATTCAACTTGCGCTCATCTTAGGC	25mer
PROBE	5'-GTTGTTGGATATGCCCTTGAC	21mer

PCR PRODUCT: 168 BASE PAIRS

REFERENCE: Svetic et al., 1991

Table 2. Oligonucleotides used in this investigation.

literature for preserved RNA sequences in PET (Stanta and Schneider, 1991). The primers are separated by two introns totaling approximately 0.2 kbp in length (Ercolani et al., 1988). Normally, this would allow rapid discrimination of amplified cDNA versus genomic DNA, either on the basis of fragment size or by adjusting PCR conditions to limit amplification to the smaller product. However, mammalian genomes carry intronless GAPDH pseudogenes (Hanauer and Mandel 1984). During PCR these segments are coamplified, along with cDNA, when genomic contamination of the preparation is present. The contamination can be distinguished through the use of appropriate control reactions. This indicates that GAPDH is potentially a suitable control for the study of RNA-PCR in PET.

Hypoxanthine phosphoribosyltransferase (HPRT) can also be used as a control in RNA-PCR (Table 2). This enzyme catalyzes an early step in the purine salvage pathway and is also constitutively expressed. The size of the PCR product is 168 bp, within the range of RNA fragment sizes extractable from PET. Because of greater than 95% homology between mouse and human HPRT cDNAs (Kim et al., 1986), this set also works in human or mouse tissue experiments (Svetic, personal communication). These primers are separated by two introns on the gene totaling 0.8 kbp and 1.5 kbp in mice and humans, respectively. This long span differentiating the genomic DNA from the mRNA derived cDNA, means that contaminating genomic DNA will not interfere with a signal generated by amplification of the cDNA (von Weizsäcker et al., 1991), nor has the existence of HPRT pseudogenes been described. This eliminates the need for a mock cDNA reaction as a control step.

Therefore, the purpose of this investigation is to determine the effects of fixation conditions on the suitability of RNA recovered from PET for use as an RNA-PCR template while comparing two control mRNAs.

MATERIAL AND METHODS

Animals: Female C3H/OuJ mice were used throughout this investigation.

Centrifugation: Multiple centrifugation steps were required in this investigation.

All 1.5 mL and 0.5 mL Eppendorf tubes were centrifuged using a TOMY MTX 150 (Tomy Seiko, Tokyo, Japan) microcentrifuge with a fixed angle rotor. All other centrifugations were performed in an IEC PR-6000 centrifuge

(Damon/IEC, Needham Heights, Massachusetts) with a swinging bucket rotor.

Unless otherwise noted, centrifugations were performed at 12000xg and 4°C.

Tissue fixation: Spleen, liver, skeletal muscle, brain and kidney were harvested from mice either immediately after the animals were sacrificed or after a 24 hour postmortem interval had elapsed. During this interval the cadavers were stored at 4°C. The tissues obtained were fixed immediately after sacrificing the animals in one of three histologic fixatives for periods of 2, 8, or 24 hours (designated 0-2, 0-8 and 0-24, respectively). At the same time an additional portion of tissue from the organs sampled was foil wrapped, frozen between blocks of dry ice and stored at -70°C to serve as the positive control. Tissues procured following the 24 hour postmortem interval underwent a 2 hour fixation (designated 24-2). Note that for each fixation time all tissues came from the same animal.

The three fixatives used in this study were; 10% neutral buffered formalin from PARA Scientific, Fairless Hills, Pennsylvania, Omnifix II from An-Con Genetics, Melville, NY and Carnoy's fixative consisting of 60% (vol/vol) absolute ethanol, 30% chloroform, and 10% glacial acetic acid (Luna, 1968). Carnoy's fixative was prepared in the laboratory from reagent grade chemicals.

Abbreviations for fixatives and fixation times used in tables, illustrations and text are summarized in Table 3.

Designation:	Definition:
0-2	0 time after death, 2 hour fixation
0-8	0 time after death, 8 hour fixation
0-24	0 time after death, 24 hour fixation
24-2	24 hours postmortem (at 4°C), 2 hours fixation
F	10% neutral buffered formalin
O	Omnifix II
C	Carnoy's fixative
Fr	Frozen tissue
P	Primers and water only control

Table 3. Abbreviations and designations used in tables, illustrations and text.

Tissue processing: Upon culmination of the desired fixation period, the tissue was immediately processed for paraffin embedding using a commercial automated processor (Tissue-Tek VIP 1000, Miles Laboratories, Elkhart, IN). Processing was accomplished without exposure of the tissue to formalin. When the processing cycle was finished, the tissue samples were embedded in standard fashion in histologic grade paraffin material (Paraplast X-tra, Sherwood Medical, St. Louis, MO) using a Histo-Center II embedder (Fisher Scientific, Pittsburgh, PA). The paraffin blocks were stored at room temperature for 1-4 weeks until used. At the end of this step, multiple sample sets of tissues were ready for further study. A sample set was defined as 12 samples of the same tissue each fixed in one of three fixatives for one of four time periods (0-2, 0-8, 0-24, or 24-2 hours) plus a frozen sample. Figure 1 is a flow diagram representing the tissue fixation/processing scheme utilized in this study.

Histologic examination: In order to compare the suitability of the three fixatives as tissue preservatives for histologic evaluation, kidney tissue from the 8 hour fixation period was embedded in three separate paraffin blocks. Tissue sections were cut from these blocks at 5 micron thickness, lifted onto glass slides and stained with hematoxylin and eosin in standard fashion. The slides were evaluated with light microscopy for adequacy of histologic preservation. Three pathologists, blinded as to the fixative used, rated the slides as adequate or inadequate for histologic interpretation.

RNase precautions: Beginning with the deparaffinization step, standard protocols against RNase contamination were observed throughout the investigation. Sterile polypropylene or glass tubes and RNase-free reagents were used. If necessary, fluids such as water were rendered RNase free by double filtration through 0.22 μ m Millex-GS filters.

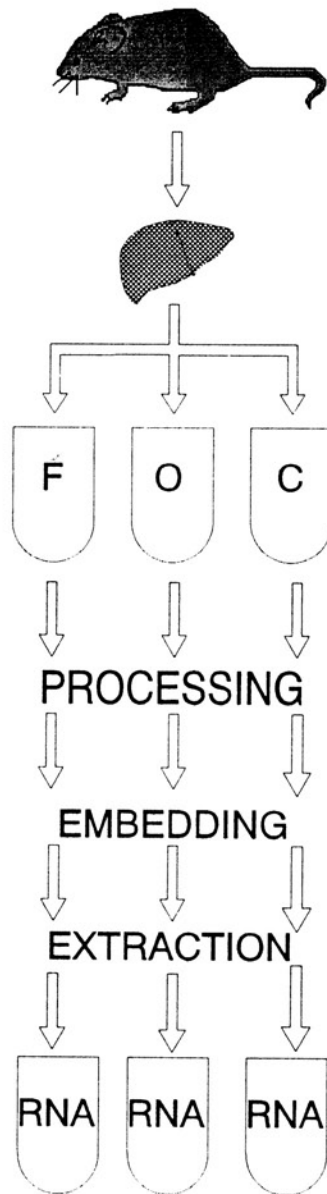


Figure 1. Flow diagram of the tissue fixation and processing scheme utilized. Only a single fixation time period is illustrated.

Deparaffinization: Deparaffinization was based on the technique of von Weiszäcker et al., (1991). Using a sterile scalpel blade, 20 mg tissue blocks representing 0.2-0.3 cm cubes were cut from the paraffin blocks. The tissue, trimmed of excess paraffin, was placed in 1.5 mL Eppendorf tubes with 1 mL of xylene. The tubes were vortexed vigorously for 30 seconds and centrifuged for 15 minutes. The xylene was decanted and this step was repeated once. After decanting the xylene for the second time, the tissue was washed with 100% ethanol. One mL of ethanol was added to the tubes followed by 30 seconds of vortexing and centrifugation for 15 minutes. The alcohol was decanted and this step was repeated once. After decanting the second alcohol wash, the tubes were dried by inverting them over tissue paper.

RNA extraction: The RNA extraction procedure used in this study was based on the manufacturer's recommendation for RNAzol B (Biotecx/Tel-Test, Friendswood, TX; Chomczynski, 1991). (RNAzol B is a proprietary guanidinium/phenol based reagent formulated for the one-step isolation of total cellular RNA from fresh or frozen tissue; Chomczynski and Sacchi, 1987).

Deparaffinized tissue was transferred to conical 15 mL tubes in a 5 mL volume of RNAzol B. The volume of RNAzol B utilized was 10-12 times in excess of the RNAzol:tissue ratio of 2 mL/100 mg of tissue suggested by the manufacturer because it was found that the additional liquid aided the homogenization process. The tissue was thoroughly homogenized in the RNAzol B using a mechanical homogenizer (Tissuemizer, Tekmar, Cincinnati, Ohio) with a stainless steel probe. Homogenization was also facilitated by manually crushing the tissue between the probe and the wall of the tube. In order to prevent carry-over between sequential samples, the probe was washed between samples, twice with sterile distilled water and once each with ethanol and RNAzol B. At this point in the procedure the frozen tissue sample was included.

Frozen samples were homogenized after the PET samples. The resultant mixture was kept on ice for 15 minutes. After 15 minutes, the homogenized samples were divided equally between two 5 mL centrifuge tubes (≈ 2.5 mL/tube). Then 250 μ L of a 49:1 chloroform:isoamyl alcohol solution was added to each of these tubes and mixed thoroughly by brief vortexing. This 250 μ L was based on a proportion of 0.1 volumes of the homogenate present. The tubes were held on ice for 5 minutes and then centrifuged for 20 minutes. Addition of the chloroform:isoamyl alcohol mixture to the RNazol B homogenate causes it to separate into two layers, a lower chloroform-phenol phase and an upper aqueous phase. RNA is present in the aqueous phase, while protein and DNA are relegated to the interphase and the lower phase (Chomczynski, 1991). Following centrifugation, a sharply demarcated clear to slightly yellow tinged aqueous phase was present above a blue lower phase.

If, after separation, any sample demonstrated a cloudy or off color aqueous phase, it was reextracted. The aqueous phase of the offending sample was transferred to a fresh 5 mL tube along with 1 mL of RNazol B and 1 mL of chloroform:isoamyl alcohol. After vortexing, the centrifugation step was repeated. The reextraction step was rarely deemed necessary.

The aqueous supernatant from each sample was transferred to a 15 mL round bottom Corex tube. Transfer was accomplished by pipetting off only the upper 75-80% of the aqueous phase. Particular care was taken to avoid the interphase and the chloroform-phenol phase. (Yamaguchi et al., 1992) This yielded a total of 2.0 to 2.5 mL of liquid when the aqueous portion of both half samples were recombined in one Corex tube

RNA precipitation: An equal volume of isopropanol was added to the aqueous phases and mixed thoroughly. The tubes were stored on ice for 15 minutes and then centrifuged for 20 minutes. The supernatant was decanted and discarded,

revealing a barely visible whitish precipitate of RNA at the bottom of the tubes. The precipitate was washed with 1.5 mL of chilled 75% ethanol by vortexing briefly, centrifuging for 15 minutes and decanting the alcohol. After the ethanol wash, the pellets were air dried by placing the tubes in a horizontal position on a laminar flow hood vent.

Next, a sodium acetate-ethanol precipitation was performed. The dried pellets were dissolved in 400 μ L water to which was added 2.5 volumes (1000 μ L) of 100% ethanol and 0.1 volumes (40 μ L) of 3M sodium acetate. This solution was divided equally between two 1.5 mL Eppendorf tube and placed in -70°C storage. After 60 minutes, one of the two tubes from each sample was removed and centrifuged for 20 minutes. The second half of each sample was stored at -70°C for future use. Following centrifugation, the RNA precipitate formed a minute white-yellow pellet. The pellet was washed with 800 μ L of 75% ethanol and centrifuged for 15 minutes. This wash was decanted and the tubes were dried in a low speed centrifugal vacuum sample drier. After final drying all PET derived samples were reconstituted in 25 μ L water, frozen tissue samples in 50 μ L. Solubilization was enhanced by placing the tubes in a 70°C waterbath for 10 minutes. Any RNA samples not utilized immediately were stored at -70°C .

DNase treatment: Selected portions of this protocol required RNA sample sets that had been DNase treated. For these sets the previously reserved second half of the samples was retrieved from -70°C storage. The use of the reserved half samples allowed for a direct comparison of DNase treated and untreated samples derived from a single extraction step. The RNA sodium acetate-ethanol precipitate was pelleted and washed as described above and reconstituted in 20 μ L of water. Two μ L of 10X DNase buffer (500mM Tris.Cl, pH 8.0, 20 mM MgCl_2) was added to the RNA along with 1 μ L of RNase-free bovine pancreatic DNase I (40 U/ μ L, Boehringer Mannheim, Mannheim, Germany). This mixture was

incubated at room temperature for 30 minutes. The reaction was stopped by the addition of 0.1 volumes of 20 mM EDTA. The RNA in the samples was then reextracted by adding 125 μ L of RNAzol B and 200 μ L of chloroform:isoamyl alcohol. After vortexing, the tubes were kept on ice for 5 minutes and centrifuged for 20 minutes. The aqueous phase, \approx 100 μ L, was then transferred to a fresh Eppendorf tube. One volume of isopropanol was added and the tubes were again placed on ice for 15 minutes and centrifuged for 20 minutes. The supernatant was discarded and the pellets were washed with 200 μ L of 75% ethanol and vacuum dried. Final reconstitution was in 25 μ L water; 50 μ L for frozen samples. A sodium acetate-ethanol precipitation step was not performed.

RNA quantitation: The RNA concentration in the final preparation was quantitated spectrophotometrically. Five μ L of RNA solution were mixed with 500 μ L of water. This was transferred to a glass cuvette and the sample's absorbance at 260 nm and 280 nm was measured with a Beckman DU-7 spectrophotometer (Palo Alto, California). The RNA concentration was calculated using the assumption that an absorbance of 1.0 at 260 nm equals 40 μ g/ μ L of RNA. As a measure of RNA purity, the A_{260}/A_{280} ratios were calculated (Sambrook et al., 1989).

cDNA preparation: The uncoupled cDNA reaction used in this protocol is a modification of the procedure described by Yamaguchi et al., (1992). A volume of the final RNA preparation ranging from 1.0 to 10.0 μ L was placed in a fresh 1.5 mL Eppendorf tube. This volume was then adjusted, if necessary, to a total of 10 μ L by the addition of water. The total quantity of RNA used ranged from 1.0 to 2.2 μ g per tube. Since these experiments were not quantitative in nature, no attempt was made to exactly equilibrate the amount of RNA from tube to tube. However, within most sample sets variation was limited to 0.2-0.3 μ g. Two μ L of random hexamers (.33 μ g/ μ L) and 1 μ L of 5X reverse transcriptase buffer

(250 mM Tris.Cl pH 8.3, 375 mM KCl, and 15 mM MgCl₂) were added. The tubes were heated to 70°C for 10 minutes to denature the nucleic acids and cooled on ice for 5 minutes. After cooling, 4 µL of 5X reverse transcriptase buffer, 2.5 µL of dithiothreitol (0.1 M), 1.5 µL of dNTP (10 mM), 1.5 µL of water and 2.5 µL of Moloney murine leukemia virus reverse transcriptase (200 U/µL; GIBCO/BRL, Gaithersburg, Maryland) were placed in each tube. Final reaction volume was 25 µL. For each cDNA reaction tube, an identical tube containing the same amount of RNA was used for a control reaction. In the negative control cDNA (mock reverse transcriptase) reaction, 2.5 µL of water were substituted for the reverse transcriptase. The tubes were incubated at 37°C for 45 minutes. After incubation, the reaction was terminated by heating the tubes to 95°C for 10 minutes. This was followed by 15 minutes of centrifugation to pellet the protein. The cDNA preparations were stored at -70°C until needed.

Polymerase chain reaction: The oligonucleotide primers used for the amplification of cDNAs during the PCR step of this protocol are depicted in Table 2. Primer selection criteria was discussed earlier. The oligonucleotides, including the probes, were synthesized with an Applied Biosystems DNA synthesizer (Foster City, California) at USUHS. Oligonucleotide purity was confirmed by polynucleotide kinase labeling and sequence gel analysis. PCR was carried out in a 100 µL reaction volume in GeneAmp tubes (Perkin Elmer Cetus, Norwalk, Connecticut). The cloned thermostable DNA polymerase from *Thermus aquaticus* (Taq polymerase) supplied by Promega (Madison, Wisconsin) was used. Reaction tubes for the amplification of GAPDH cDNA were prepared with the following components: 3 µL of dNTP (10 mM), 10 µL 10X Taq polymerase buffer (500mM KCl, 100 mM Tris-HCl pH 9.0, 1% Triton X-100), 6 µL MgCl₂ (25 mM), 75 (C) µL water, 2 µL each of sense and antisense primers (0.2 µg/µL), 0.4 µL of Taq polymerase (5 U/µL) and finally, 2 µL of the cDNA

solution. When HPRT was the sequence targeted for amplification, the volume of water was decreased to 72 μ L, and 5 μ L of the cDNA was used along with the HPRT specific primer set. Since the cDNA was prepared using random hexamers, the same cDNA preparations were used regardless of the sequence being targeted. The mixes were overlaid with 100 μ L of mineral oil and run for the prescribed number of cycles using a Perkin Elmer Cetus (Norwalk, Connecticut) thermal cycler, version 2.1. The PCR cycling parameters are delineated in Table 4. The PCR parameters used were based upon those of existing cytokine gene expression protocols in current use at USUHS (Dveksler et al., 1992; Svetic et al., 1991). PET sample amplification was determined by increasing the number of cycles until positive bands could be detected with Southern blot analysis. The detection points were well below the reaction saturation points. Absence of a detectable band after the prescribed number of cycles was considered a negative result. Additional cycles beyond this point showed overamplification in the positive bands while negative bands remained so. Upon completion of thermal cycling the PCR products were stored at 4°C.

As described by Dveksler et al., (1992) each PCR experiment included a number of control reactions. For GAPDH, a parallel set of tubes in which mock cDNA reaction products substituted bona fide cDNA was simultaneously prepared and cycled as the negative control. This was done in order to test for the presence of genomic DNA contamination in the original RNA extraction. With HPRT the amplification of genomic DNA was precluded on the basis of primer design, so this step was eliminated (see introduction). All PCR experiments included a negative control consisting of all components and primers, but without cDNA. Negative results here precluded the possibility of contaminated primers, water or reagents (Dveksler et al., 1992). The frozen samples served as the positive controls to confirm that the PCR amplification and the preceding cDNA

GAPDH:

DENATURATION	94°C	1 MIN 30 SEC
ANNEALING	50°C	1 MIN 30 SEC
EXTENSION	72°C	1 MIN 30 SEC

HPRT:

DENATURATION	94°C	0 MIN 45 SEC
ANNEALING	50°C	1 MIN 00 SEC
EXTENSION	72°C	2 MIN 00 SEC

Table 4. PCR cycling parameters used for GAPDH and HPRT.

reaction had been performed satisfactorily. Thus, each PCR experiment was performed on either 27 tubes (GAPDH) or 14 tubes (HPRT) This included all PET samples of a particular tissue plus the appropriate positive and negative controls.

Gel Electrophoresis: Products of the PCR amplification of targeted cDNA sequences were fractionated by gel electrophoresis. Agarose gels were prepared by melting 3 gm of low melting point, molecular biology grade agarose (GIBCO/BRL) in 150 mL of 1X TBE (0.09 M Tris-borate, 0.001 M EDTA). Microwaving for 6 minutes, 30 seconds was sufficient to liquefy the agarose. The agarose solution was cast in 11 X 14 cm plates with 14 well combs in place. After cooling at room temperature, gels were submerged in an electrophoresis chamber containing 1X TBE. The gels were loaded with a mix of 15 μ L of PCR products and 4 μ L of 0.25% bromophenol blue. The marker consisted of 3-4 μ L of 100 base pair ladder (1 μ g/ μ L; GIBCO/BRL) in water and bromophenol blue. Electrophoresis was run at 96V for approximately 90 minutes. At the end of the run, gels were stained in a dilute solution of ethidium bromide and examined under UV illumination for the presence of bands. In general, amplification of cDNAs within the parameters of this study did not result in visible bands. Overamplification of the frozen samples, beyond 25 cycles, produced bands visible on ethidium staining. This occasionally proved useful in confirming a successful PCR experiment.

Southern blotting: Gels were denatured by soaking in several volumes of 1.5 M NaCl, 0.5 M NaOH under constant gentle agitation for 40 minutes. Neutralization of the NaOH was accomplished by rinsing the gels with deionized water and soaking in several volumes of 1 M Tris-Cl pH 7.4, 1.5 M NaCl for 20 minutes, also under agitation. The DNA was transferred to either 0.2 or 0.45 μ m pore size nylon membranes (Nytran, Schleicher and Schuell, Keene, New

Hampshire). Transfer was performed by the capillary method of Southern (1975). Whatman 3MM paper (Hillsboro, Oregon) was wrapped around a plexiglass support and placed in a vessel partially filled by 20X SSPE (sodium chloride, sodium phosphate EDTA). The paper was allowed to saturate with the solution by capillary action. At the same time the nylon membrane, trimmed to fit the gel was pre-wetted with 20X SSPE. After neutralization, the gel was inverted and placed on the 3MM paper covered support. The membrane was placed over the gel and air bubbles were removed. Care was taken not to disturb the original position of the membrane. Two more sheets of 3MM paper were placed on the membrane, followed by a 3-5 cm stack of paper towels. These were topped off with a plexiglass plate and a 500 gm weight. Any exposed paper on the support was blocked with plastic kitchen wrap. Blot transfer was allowed to proceed overnight, approximately 14 hours. The following morning, the nylon membrane was marked for lane orientation and removed from the gel. DNA was crosslinked to the membrane with a 2 minute UV exposure in a DNA transfer lamp (Fotodyne, New Berlin, Wisconsin).

Blot analysis: Crosslinked blots were prehybridized in a buffer solution of 5X SSPE, 5X Denhardt's Solution and 0.1% SDS (sodium dodecyl sulfate). One or more membranes (blotted with the same DNA products) and 10 mL of prehybridization buffer were sealed in a bag and placed in a 42°C water bath for 90 minutes. During prehybridization, the specific oligonucleotide probe, either GAPDH or HPRT (Table 2), was radiolabeled. Labeling was carried out in a 10 μ L reaction volume. Four μ L of probe (0.2 μ L/ μ g), 3 μ L water, 1 μ L 10X kinase buffer (700 mM Tris-HCl pH 7.6, 1 M KCl, 100 mM MgCl₂, 50 mM dithiothreitol), 0.5 μ L of T4 polynucleotide kinase (10 U/ μ L; GIBCO/BRL) and 1.5 μ L of γ [³²P]-ATP (75 μ Ci/ μ L; New England Nuclear, Boston, Massachusetts) were mixed in a 500 μ L reaction tube. The reaction was incubated at 37°C for 30

minutes and then stopped with the addition of 2 μ L 0.5 M EDTA. After 90 minutes had elapsed, the hybridization bags were slit and drained. Probe solution mixed with 10 mL of the prehybridization solution was added to the bags and allowed to hybridize for 3 hours in a 42°C water bath. When 3 or more blots were being hybridized, 6 μ L of probe solution was used, otherwise 3 μ L was sufficient. At the end of the hybridization phase the blots were removed and washed by agitation in several volumes of 2X SSPE, 0.1% SDS at room temperature. This wash was repeated four times or until cpm in the discarded wash had reached background levels. A final, higher stringency wash in 5X SSPE, 0.1% SDS was performed at 44°C for 20 minutes. The washed, hybridized blots were dried between paper towels and wrapped in plastic wrap. The blots were autoradiographed on XAR-5 film (Kodak, Rochester, New York) with a Kodak enhancement screen. Autoradiography was performed overnight at -70°C.

When developed, the autoradiographs were examined for the presence of hybridized bands. Any observable band of the correct length was considered a positive result. In some instances, if weak bands were thought to be present, the blots were reexposed overnight to phosphor screens and evaluated with the Phosphor Imager using Image Quant software (Molecular Dynamics, Sunnyvale, California). Once again, detectable bands were interpreted as positive results.

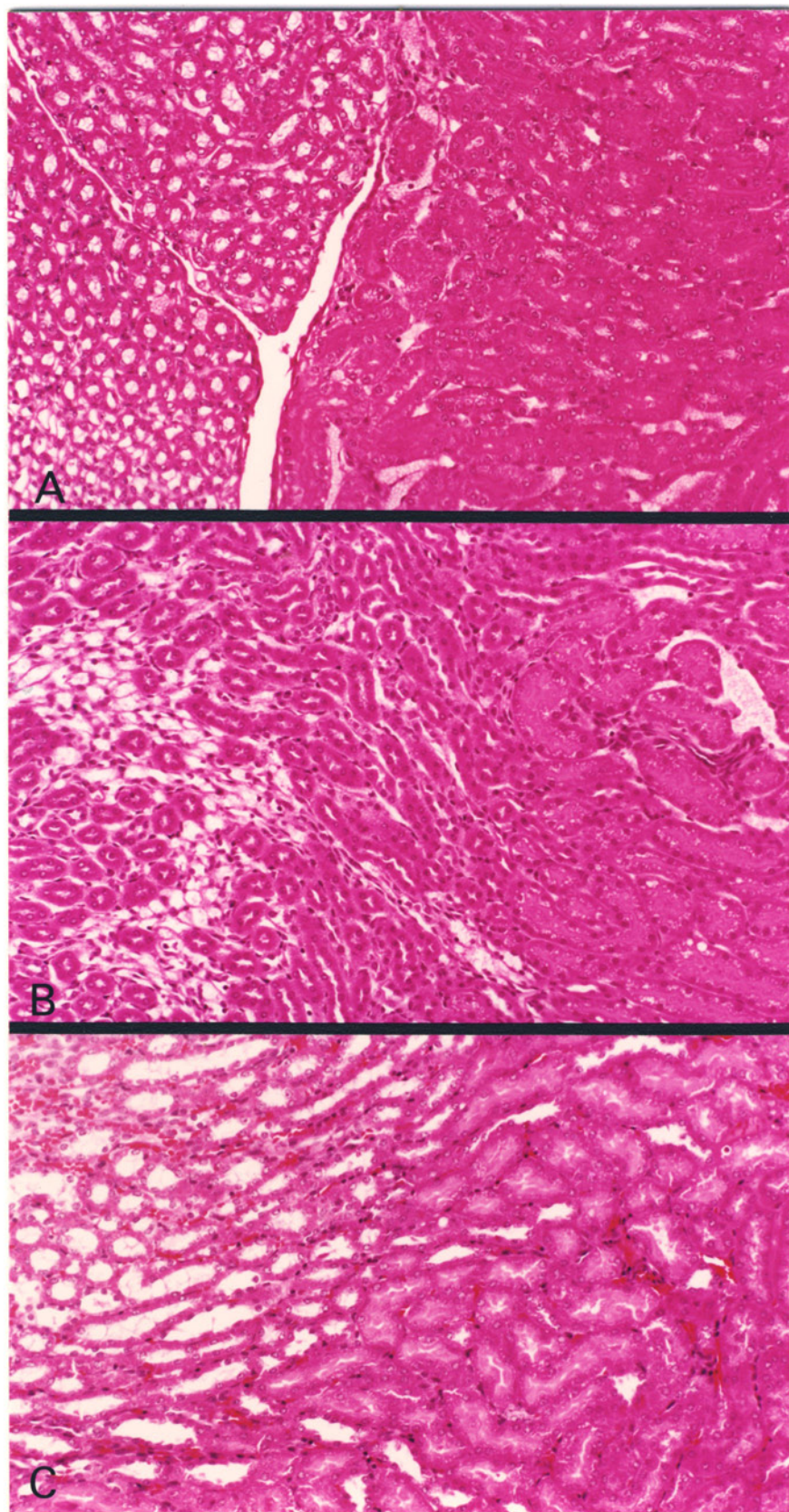
RESULTS

Histology: Histologic sections of renal tissue from the 0-8 hour fixation period were deemed to be of acceptable interpretive quality for all three fixatives. The three blinded observers subjectively favored either Omnifix II (2 observers) or Carnoy's fixative (1 observer) as providing optimal results. Interestingly, none of the observers thought that tissue fixed in 10% neutral buffered formalin produced the best results. Lysis of red blood cells was present in the Omnifix II and Carnoy's fixed samples. Representative histology of each specimen is illustrated in Figure 2.

RNA extraction: All PET samples yielded extractable RNA using the RNeasy B technique. RNA yields for different tissue types are summarized in Table 5. Minor variations in yields were observed among different fixatives and times, but no particular fixative, time or combination thereof provided significantly higher or lower yields on a consistent basis. PET yields ranged from 10-20 fold lower than frozen tissue for liver and spleen and 2 fold lower than frozen brain and muscle. Despite the differences in the amount of yield reduction, final RNA yields were in the same range for all PET samples. Yields from the frozen tissues, extracted in parallel with the PET samples, were consistent with those reported by the manufacturer (Chomczynski, 1992). The observation of appropriate results for frozen yields (Table 5) indicates that the extraction procedure was performed correctly.

In those instances in which it was necessary to reextract the supernatant, no appreciable differences were noted in RNA yield when compared to the samples extracted only once.

Figure 2. Histologic comparison of renal tissues fixed for 8 hours in; (A) 10% neutral buffered formalin, (B) Omnifix II and (C) Carnoy's fixative. Tissues were processed, embedded and stained concurrently. Hematoxylin and eosin stain; magnification 50X.



<u>FIXATIVE</u>	<u>TIME</u>	<u>RNA YIELD (µg/mg tissue)</u>			
		<u>Spleen</u>	<u>Liver</u>	<u>Muscle</u>	<u>Brain</u>
Formalin	0-2	0.5	0.8	0.6	0.5
Omnifix II	0-2	0.5	0.7	0.6	0.8
Carnoy's	0-2	0.5	1.1	0.6	0.9
Formalin	0-8	0.5	0.6	0.6	0.7
Omnifix II	0-8	0.5	2.3	0.9	0.9
Carnoy's	0-8	0.5	1.5	0.6	0.8
Formalin	0-24	0.5	0.5	0.4	0.8
Omnifix II	0-24	0.7	1.3	0.4	0.5
Carnoy's	0-24	0.5	0.6	0.6	0.5
Formalin	24-2	0.5	0.7	0.6	0.3
Omnifix II	24-2	0.6	0.5	0.6	0.5
Carnoy's	24-2	0.5	1.0	0.6	0.4
Frozen	N/A	6.0	8.0	1.7	1.4
Predicted range for frozen tissue (Chomczynski, 1992)		7-10	7-10	1-1.5	1-1.5

Table 5. RNA yields from PET using the modified RNazol B extraction method. The values for spleen represent the mean of 2 extractions.

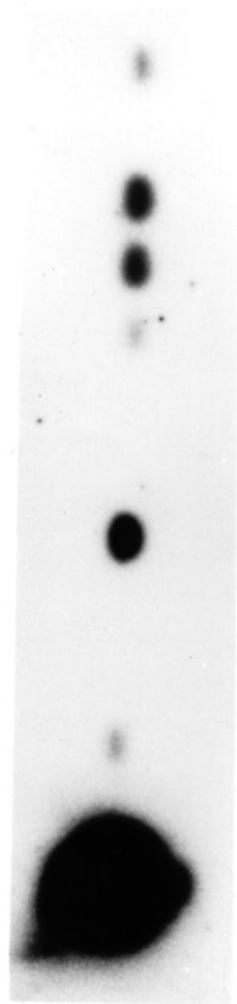
The purity of RNA extracted from PET using this technique was acceptable for all samples. The A_{260}/A_{280} ratios of the PET samples generally exceeded the results predicted on the basis of the manufacturer's literature, while the frozen controls were within the published range (Chomczynski, 1991, Yamaguchi et al., 1992). The absorbance ratios for the tissues sampled are summarized in Table 6. As with RNA yield, some variation among individual samples was present, particularly in the brain set, but no trends with respect to fixation or time were observed. Previous studies have demonstrated that RNA preparations with absorbance ratios above 1.4 are adequate for RNA-PCR experiments (Yamaguchi et al., 1992). All samples evaluated in the current protocol were well above that level and considered to be usable for PCR based on this measure. Comparison of the absorbance ratios between those samples extracted once with RNazol B and the ones extracted twice did not reveal any significant differences.

cDNA Preparation: The random hexamer primed cDNA reaction methodology utilized in this investigation proved to be rapid and efficient. This technique provided sufficient cDNA for multiple PCR procedures and, if necessary, allowed the amplification of multiple different sequences from the product of a single reaction. Figure 3 shows a single cDNA preparation used for the amplification of two unrelated RNA species. Although the GAPDH PET samples are negative, the frozen controls for GAPDH and HPRT are both positive. The mock reverse transcriptase reaction, in which water was substituted for the reverse transcriptase, proved a sensitive negative control for RNA-PCR by reliably detecting the presence of DNA contamination when the GAPDH primers were used.

<u>FIXATIVE</u>	<u>TIME</u>	<u>A₂₆₀/A₂₈₀ RATIOS OF EXTRACTED RNA</u>			
		<u>Spleen</u>	<u>Liver</u>	<u>Muscle</u>	<u>Brain</u>
Formalin	0-2	2.51	2.33	2.94	3.53
Omnifix II	0-2	2.52	2.28	2.85	2.17
Carnoy's	0-2	2.50	2.22	2.71	1.82
Formalin	0-8	2.63	2.54	2.80	2.09
Omnifix II	0-8	2.56	1.94	2.37	2.02
Carnoy's	0-8	2.72	2.09	2.67	1.88
Formalin	0-24	2.44	2.53	3.18	2.15
Omnifix II	0-24	2.39	2.21	2.85	1.76
Carnoy's	0-24	2.61	2.46	3.05	1.50
Formalin	24-2	2.54	2.52	3.17	2.64
Omnifix II	24-2	2.45	2.74	3.05	1.91
Carnoy's	24-2	2.54	2.34	3.00	2.16
Frozen	N/A	1.67	1.69	1.81	2.00
Predicted range of frozen tissue ratios (Chomczynski, 1991)		1.6-1.9	1.6-1.9	1.6-1.9	1.6-1.9

Table 6. A₂₆₀/A₂₈₀ ratios of RNA extracted from PET using the modified RNAzol B method. The values for spleen represent the mean of 2 extractions.

Figure 3. PCR amplification of unrelated sequences from a single, random hexamer primed, cDNA reaction. Reverse transcription was performed on a DNase treated splenic RNA preparation. Part A is HPRT, part B is GAPDH. In the PET samples, GAPDH activity has been abolished by DNase treatment. PCR cycles: HPRT frozen, 25; HPRT PET, 35; GAPDH frozen, 20; GAPDH PET, 30. The frozen HPRT sample is overamplified at 25 cycles. See Table 3 for an explanation of symbols.



A



B

Fixative Time	P	Fr	0-2			0-8			0-24			24-2		
			F	O	C	F	O	C	F	O	C	F	O	C

RNA-PCR: With a limited exception, it was not possible to detect amplifiable GAPDH RNA from the PET samples. Initial PCR amplifications of GAPDH cDNA indicated extensive contamination with genomic DNA. This was indicated by the presence of a 195 bp band on Southern blots of the mock cDNA (negative control) PCR products. Figure 4 shows the results of a representative example of amplified splenic RNA before and after DNase treatment. Brain and liver showed similar patterns of contamination. The DNA contamination was not masking viable RNA amplification because, as illustrated, DNase treatment abolished all GAPDH reactivity in the samples. The exception to this finding was in muscle, a tissue which expresses high levels of GAPDH. In Figure 5, valid bands are shown to be present for the 0-2 and 0-8 Carnoy's fixed muscle samples after DNase treatment has eliminated the contaminating DNA. Frozen controls performed as expected and did not demonstrate evidence of DNA contamination in the prescribed amplification range of 20 PCR cycles (Yamaguchi et al., 1992). These results indicate very limited preservation of intact GAPDH mRNA sequences over 195 bp long in PET. Only immediate fixation of muscle in Carnoy's fixative for 2 or 8 hours yielded amplifiable RNA sequences.

In contrast to GAPDH, amplification of the 14% shorter 168 bp HPRT segment provided better evidence of preserved mRNA sequences in PET. Since the primers selected spanned two introns and PCR parameters were chosen to preclude amplification of the longer genomic HPRT segment, DNA contamination was not a factor in the HPRT reactions. Since only amplification from the RNA template would result in a 168 bp product, the valid bands could be readily identified.

Figure 4. RNA-PCR amplification of GAPDH from splenic RNA before and after DNase treatment. A and B show the samples prior to DNase treatment. Part A represents the mock reverse transcriptase reaction. The bands present indicate that contaminating DNA has been amplified. Part B is the corresponding cDNA positive reaction. C and D show the samples after DNase treatment. Part C is the autoradiograph of the mock reverse transcriptase reaction and D is the corresponding cDNA positive blot. The RNA used in A, B, C and D is from the same extraction (see Materials and Methods). PCR cycles: frozen, 20; PET, 30. See Table 3 for an explanation of symbols.

Figure 5. RNA-PCR amplification of GAPDH from paraffin embedded muscle tissue. Part A, the mock reverse transcriptase reaction products before DNase treatment, indicates DNA contamination in all the Carnoy's fixed samples. Note that the weak band in lane Fr (frozen) is the result of overamplification at 30 cycles. Part B shows the mock reverse transcriptase reaction products after the samples have been DNase treated. C is the cDNA positive blot after DNase treatment. PCR cycles: frozen, 20 (except A); PET, 30. See Table 3 for an explanation of symbols.

Figure 6 shows that a total of 40% of the amplifications for HPRT were positive, 19% of those fixed in formalin and 50% of those fixed in Omnifix II and Carnoy's fixative. There is clearly better preservation of RNA by non-formalin based fixatives. Of samples fixed immediately after death, Omnifix II fixation (9/12 positive), was superior to Carnoy's and formalin fixation with 4/12 and 0/12 positive, respectively. The fixation time providing the greatest number of positive results was 8 hours followed closely by 24 hours and then 2 hours. The results for 2 hours of fixation following a 24 hour postmortem interval are somewhat anomalous when compared to immediate fixation. In this group 3/4 formalin fixed and 3/4 Carnoy's fixed samples had positive bands while Omnifix II fixation produced no bands. In comparison, no positive bands were identified for either Carnoy's fixative or formalin in the immediate 2 hour (0-2) fixation group in 4 separate amplification runs. Figure 6 illustrates three representative Southern blots of HPRT amplification products.

For both GAPDH and HPRT, controls for contamination of primers with PCR products or template cDNA were used (Dveksler et al., 1992). These controls, consisting of a PCR mix with sense and antisense primers plus water instead of template cDNA, were uniformly negative.

DNase Treatment: The results for GAPDH showed that RNase free DNase I treatment of the samples was a rapid and effective means of eliminating DNA contamination as shown in Figure 4.

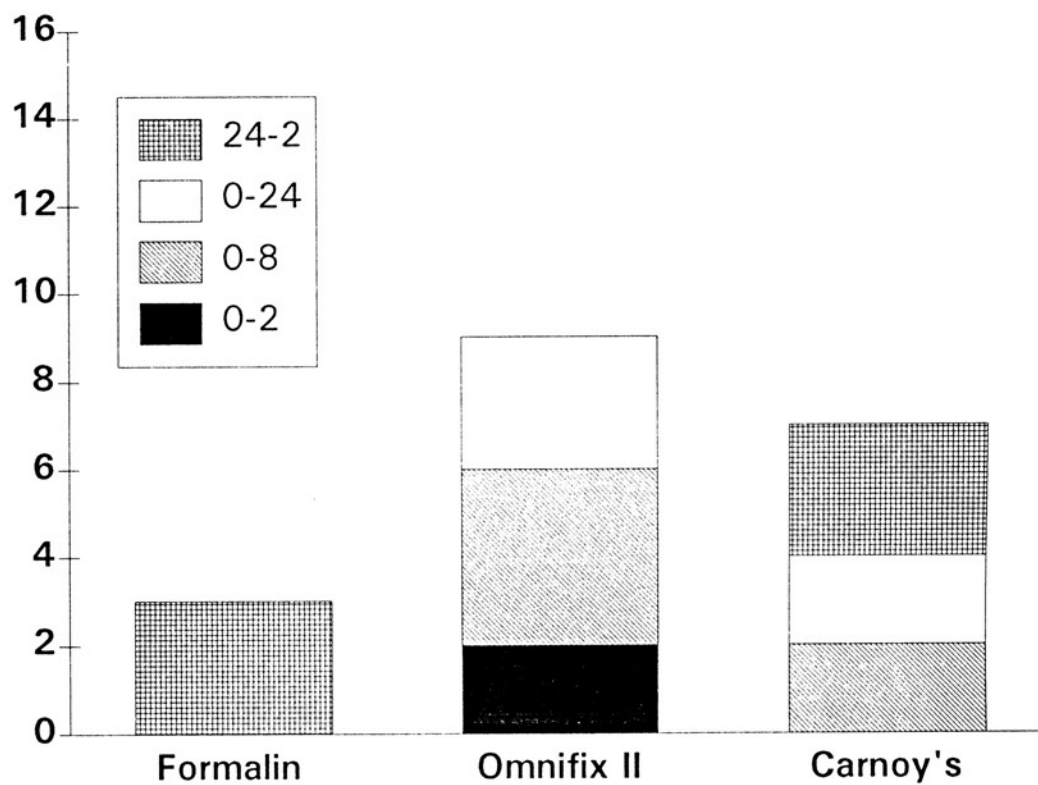
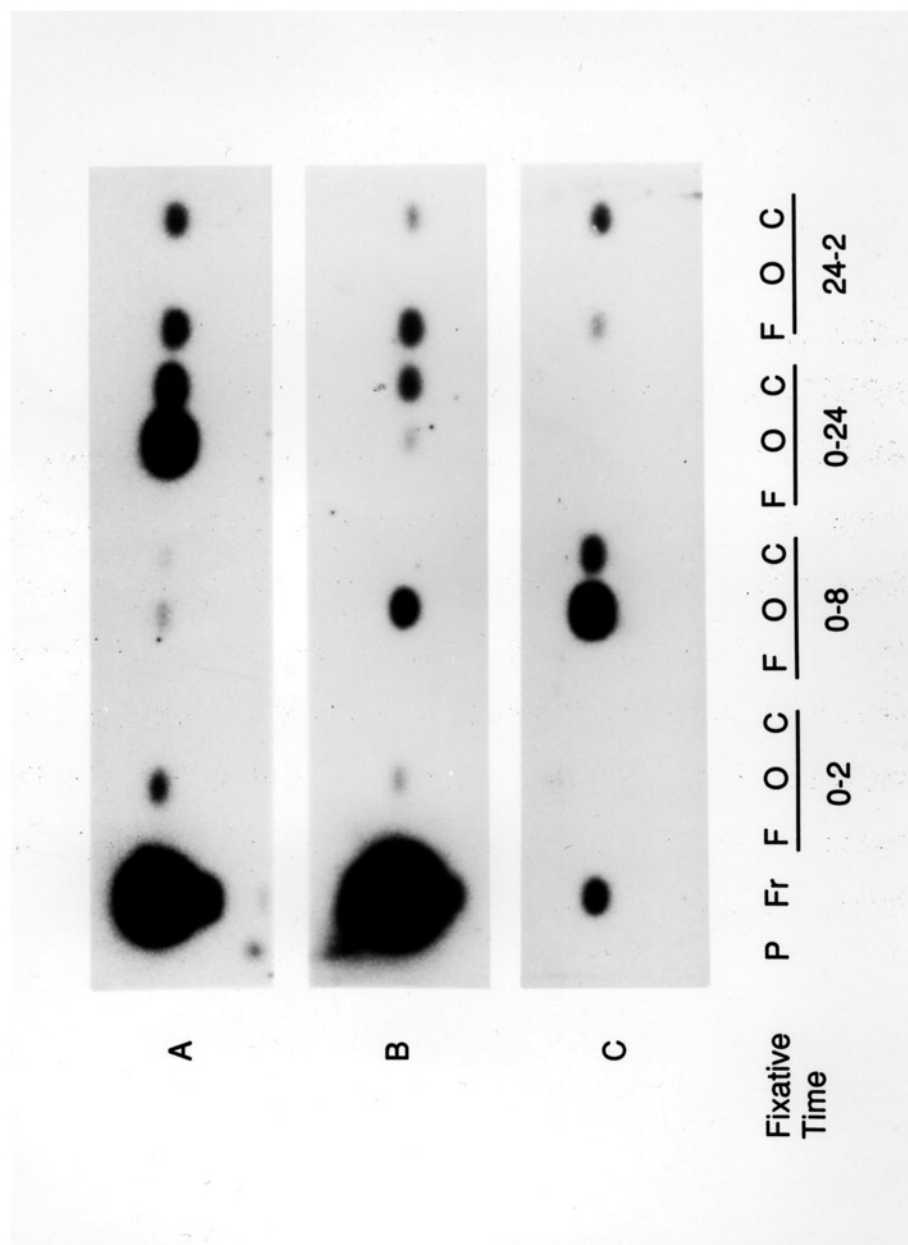


Figure 6. Graphic representation of HPRT positive results by fixation type. Sixteen is the number of PCR procedures performed for each fixative. See Table 3 for an explanation of fixation time symbols.

Figure 7. Representative autoradiographs of Southern blots of HPRT RNA-PCR amplification products. Part A is from splenic RNA which was not DNase treated. Part B is from a different preparation of splenic RNA which was DNase treated. Part C is from brain RNA which was not DNase treated. Parts A, B, and C are from separate RNA extractions and the amounts of RNA used were not uniform (see Materials and Methods). Therefore, comparisons between band intensities should not be made. PCR cycles: frozen spleen, 25; frozen brain, 20; PET, 35. See Table 3 for an explanation of symbols.



DISCUSSION

This investigation evaluated the effects of fixation and paraffin embedding on the extraction and subsequent PCR amplification of PET derived RNA. The variables evaluated were three specific histologic fixatives, time of fixation and the effect of a postmortem interval.

Fixatives: Three fixatives, 10% neutral buffered formalin, Omnifix II and Carnoy's fixative, were selected for comparison. Several factors influenced the choice of these three. Each one has been purported to provide optimal nucleic acid preservation in a separate previous study (Jackson et al., 1990; Greer et al., 1991; Ben-Ezra et al., 1991) which provided a rationale for their comparison. They are available commercially or are readily prepared from basic laboratory reagents and provide satisfactory histologic results. Obviously, a fixative unsuited for histologic evaluation would be of limited value in a clinical laboratory setting. Although there are numerous other fixatives available, the majority are related to one of the fixatives in this group. This provides a basis for the extrapolation of the fixative results in this study. The acetone based AMeX method is lengthy and requires special accommodation without offering any advantage in RNA preservation (Sato et al., 1986; Sato et al., 1991). Picric acid and mercuric chloride based fixation have been shown to be relatively unsuitable for subsequent PCR analysis (Greer et al., 1991) and were therefore excluded.

Procurement of PET for analysis: In this investigation, cubes of tissue were cut directly from the paraffin blocks with sterile blades used only once. This is in contrast to the use of microtome sections in other investigations (Jackson et al., 1989; Stanta and Schneider, 1991) and offers a number of advantages over that

technique. The risk of carryover between samples and the need for cumbersome decontamination steps are eliminated. For the purposes of analysis, this method provides more precise control over tissue mass and volume than is possible using microtome sections (Jackson et al., 1990). Also, sufficient tissue volume for multiple amplification reactions can be rapidly obtained. Under the conditions described in the Materials and Methods section, 20 mg of PET contained sufficient extractable RNA for 10 or 24 PCR procedures for HPRT and GAPDH, respectively. When using microtome sections and a single tube for the extraction, reverse transcription and amplification steps, there is no provision for amplifying both control and experimental RNA from the same preparation (Ben-Ezra et al., 1991; Stanta and Schneider, 1991). When the blade/cube method is applied to actual surgical pathology material, areas of neoplastic tissue can be cut from the tissue blocks, avoiding adjacent normal tissue. Non-neoplastic tissue in microtome cross sections may skew the results of RNA or DNA analysis.

Extraction of RNA from PET: A portion of this study was dedicated to the evaluation of a method for the rapid extraction and purification of cellular RNA from paraffin embedded tissues. The RNeasy B, one step extraction technique was adapted for this usage. Utilizing this methodology, quantities of RNA sufficient for analysis can be readily obtained. The yield of RNA did not vary significantly from one type of tissue to another or with the fixation type, although frozen control tissue yields did vary as expected. Although few previous studies of RNA extraction from PET have specified the yields obtained, this technique compares favorably with those that have been reported. Rupp and Locker (1988) recovered approximately 1.0 µg/mg of RNA from formalin fixed, paraffin embedded liver while Sato et al., (1991) extracted a mean of 0.66 µg/mg of RNA from AMeX processed liver tissue. In this study, the RNeasy B process provided

an average yield of 1.0 µg/mg from paraffin embedded liver in a shorter time period and with fewer steps. The major advantage of this technique is that it eliminates the need for a lengthy proteinase K digestion step. Digestion can add 6 or more hours to an extraction procedure without improving the yield or purity (von Weizsäcker et al., 1991; Finke et al., 1993). Using the RNazol B based procedure, template RNA could be prepared from paraffin blocks in under 3 hours. The purity of this RNA, as measured by the A_{260}/A_{280} ratio, is well above the minimum necessary for successful PCR applications. As a point of interest, it should be noted that yields from frozen tissue were approximately 10 fold greater using RNazol B than they were in the study by Sato and colleagues (1991).

DNA contamination: DNA contamination of the RNA preparation represents a widely reported finding in studies evaluating RNA extraction from PET (Rupp and Locker, 1988; Ben-Ezra et al., 1991; von Weizsäcker et al., 1991). The current investigation corroborated this observation. The mechanism by which this contamination occurs is unclear, particularly since DNA did not present a problem in the frozen tissue extracted by the same method. This could possibly be explained as the result of chemical and mechanical changes in the nucleic acids or of alterations in nucleic acid-protein interactions. The formaldehyde in formalin does react with the nucleic acids (Haselkorn and Doty, 1961), but since similar contamination was present with both formalin and non-formalin based fixatives this is not likely to be the primary mechanism. Contamination of the RNA preparation may also be related to the fragmentation and subsequent decrease in the molecular weight of both DNA and RNA that occurs during tissue fixation.

Low levels of DNA contamination may or may not be a critical factor in RNA experiments. In some cases, such as HPRT, judicious selection of primers

and PCR parameters can nullify the influence of contaminating DNA. However, when this is a concern, such as when genomic DNA interferes with signals generated by cDNA amplification, use of the GAPDH primer set described in this investigation acts as a sensitive indicator. It has been demonstrated that genomic DNA, when present, can be eliminated by a brief DNase I treatment. Reverse transcription: Utilization of a random hexamer primed cDNA reaction allows a high degree of flexibility in subsequent PCR analysis of the material. This is because more than one RNA species can be amplified from the same extraction preparation. This may increase the validity of making direct qualitative comparisons between two messages from the same tissue.

Housekeeping gene controls for RNA-PCR: For the purposes of this investigation, probe and primer sets amplifying and detecting mRNA from the housekeeping genes GAPDH and HPRT were used to evaluate the integrity of the extracted RNA. These messages were chosen because they are well established as internal controls for qualitative and semiquantitative RNA-PCR experiments on fresh and frozen tissues (Murray et al., 1990; Dveksler et al., 1991; Svetic et al., 1991). Only two muscle tissue samples generated valid positive bands for GAPDH when the reaction products were Southern blotted. Because of the relative failure to successfully amplify GAPDH mRNA sequences and the interference from DNA contamination, the GAPDH primer set is of limited value as a control in experiments using RNA from PET.

A notably broader range of samples was positive for HPRT than for GAPDH. Failure to detect targeted RNA in a given sample must be attributed to RNA degradation and fragmentation during fixation since both genes are constitutively expressed and frozen controls were positive. This being the case, and since the targeted sequence of HPRT is 27 bp shorter than the 195 bp GAPDH sequence, it can be postulated that the length of the target RNA is a

critical factor in successful PCR. The concept of a critical fragment length of around 200 bp is supported by Northern blot analysis demonstrating that the fragmentary RNA extracted from formalin fixed PET averages this size or below (Stanta and Schneider, 1991). Certainly, Stanta and Schneider (1991) achieved a high success rate on formalin fixed PET using a relatively short sequence of the retinoblastoma gene cDNA, but this sequence is not established as a reliable control. If the use of PET for RNA-PCR experimentation was limited simply by restricting the choice of targeted sequences to those in the 150 bp and below range, the technique would still provide a great deal of versatility to the investigator. The results indicate that HPRT represents an ideal choice as a housekeeping gene control for such studies.

Fixation considerations: The results indicate that recoverable, PCR substrate quality RNA was greatest in Omnifix II and Carnoy's fixed samples clustered around the 8 hour fixation period. This suggests that an optimal fixation time lies in this range. An 8 hour fixation period is not impractical to consider since it correlates well with a normal workday. Results from the simulated autopsy samples which were fixed for two hours after a 24 hour postmortem interval, are somewhat anomalous. In this group, formalin and Carnoy's fixed but not Omnifix II fixed samples produced positive bands. It is unclear why delaying fixation for 24 hours would produce positive bands when immediate fixation for the same time period does not. Early autolytic changes in the tissues may play a role. Other PCR studies on PET have shown less variation in the results between fixation times. In the study of the effects of fixation on DNA PCR by Greer and colleagues (1991), formalin and ethanol (similar to Omnifix II) fixatives provide similar results between 1 and 24 hours. Carnoy's fixative was only slightly less effective in the 24 hour group. In the examination of both DNA and RNA

preservation by Ben-Ezra et al., (1991) there were negligible differences between formalin and Omnifix at 6, 24 and 48 hours of fixation.

The specific mechanism of the problems associated with RNA preservation and fixation time are hypothetical. It may be that shorter fixation times are insufficient for the denaturation of endogenous RNases and permit continued degradation of mRNA. Longer fixation times possibly result in harsh chemical environments and nucleic acid degradation.

From examining the results, without consideration of fixation time, it is clear that both Omnifix II and Carnoy's fixative are approximately equal in terms of RNA preservation and both offer advantages over neutral buffered formalin. This supports the contention that the absence of formalin is more critical than pH for the conservation of intact RNA sequences.

CONCLUSION

The outcome of this investigation indicates there is probably only a small role for the application of this technique to retrospective RNA-PCR analysis of archival PET specimens of human tissue. These specimens are generally in the form of formalin fixed PET which, on the basis of these findings, probably contain minimal amplifiable mRNA sequences over 150 bp in length. Since other studies (von Weizsäcker et al., 1991; Finke et al., 1993) have had greater success with RNA-PCR on formalin fixed material, additional studies are worthy of consideration. The results of the simulated autopsy portion of this investigation also indicates the need for more study of autopsy specimens. Even if retrospective studies do not prove fruitful, there are other potential applications of this methodology. Current state-of-the-art molecular pathobiology techniques for RNA study in neoplasia are dependent on the availability of banked frozen tissues (Naber et al., 1992). While this represents an ideal situation, in reality a vast volume of formalin fixed tissue becomes available for potential study at smaller centers which can neither fiscally nor physically support such a frozen tissue facility. At centers without access to frozen tissue banking, tissues on which subsequent RNA analysis may be of interest should be fixed in either Omnifix II or Carnoy's fixative, in contrast to formalin.

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